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Gene-dose titration analysis in the search of trans-regulatory genes in *Drosophila*

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We have searched for trans-regulatory genes in two genetic systems in *Drosophila*, the bithorax complex (*BX-C*) and the achaete-scute complex (*AS-C*). Previous genetic evidence suggests that the activation of both *BX-C* and *AS-C*, depends on trans-regulatory genes (Polycomb, *Pc*, in the former and hairy, *h*, in the latter) acting in a negative type of control. Mutants of these regulatory genes in heterozygous condition have dominant derepression phenotypes in flies with extra doses of the corresponding gene complexes. We have searched for new loci, with similar gene-dose relationships. We have isolated only new alleles (six) of *Pc* in the *BX-C* experiment. In the *AS-C* experiment four *h* alleles, and 13 alleles of a new locus (extramacrochaetae, *emc*) have been discovered. Whereas the *h* locus shows specific interactions upon achaete, the new locus, *emc*, is specific for the scute part of the *AS-C*. Statistical analysis suggests that these are the only loci in the genome with those dose-dependent properties in the two systems.

Key words: cell differentiation/gene complexes/gene regulation

Introduction

In embryonic development, terminal cell differentiation is indicated by the appearance of specific gene products. There are many classical examples of enzymes with characteristic patterns of tissue and stage specificity. It is generally assumed that these specificities reflect the existence of control mechanisms. Many models have been proposed, and particular cases explained, suggesting that this regulation occurs at any step from transcription to the physiological modulation of the activity of the gene product. Genetic analysis, on the other hand, has uncovered a series of operations between mutants which could represent the counterpart of gene regulation. Some double-mutant combinations which give synergistic phenotypes, and mutants which behave as suppressors or enhancers of others are indicative of genes involved in related pathways. It is a challenge to genetic analysis to devise tests which could restrict the possible explanations of these interactions to those typical of specific trans-regulatory genes, and in doing so, to propose specific candidates for molecular analysis of these interactions.

A particular type of gene interaction looks promising in this respect. We call it "gene-dose titration analysis". It is based on studies of phenotypes of individuals in which the ratio of one gene in the genome relative to others is not, as in wild-type, 2:2 but 1:3 or 1:4. Thus, if the former is a "regulator" gene whose products are specific to the regulation of the latter ("structural") gene, there may be enough of these products to activate (or repress) the latter in the wild-type condition but insufficient in the higher gene ratios. This insufficiency of one single gene dose (haplo-insufficiency) gives rise to phenotypes typical of the repression or derepression of the structural gene.

As shown below, this is the situation between certain genes and two gene complexes relatively well studied, the bithorax complex (*BX-C*) which determines the specification of segments (Lewis, 1978) and the achaete-scute complex (*AS-C*) which affects the differentiation of cells of the central and peripheral nervous system (García-Bellido and Santamaria, 1978; García-Bellido, 1979). The purpose of the present work is to search for mutants in loci which, in the heterozygous condition, produce new phenotypes in flies carrying several doses of either of the two gene complexes. Thus, otherwise recessive mutations, induced anywhere in the genome in a mutagenesis experiment, should be revealed in the F_1 because of this dominant, haplo-insufficient, phenotype. We expect to find new alleles to those genes already known to have "trans-regulatory" effects upon either of the gene complexes, and also to find new loci, if they exist, with similar interactions.

Specific antecedents

Genetic data relevant to the regulation of both *BX-C* and *AS-C* complexes are summarized here (see Capdevila and García-Bellido, 1981; García-Bellido, 1981a, for discussion and references). They suggest that both systems are under a negative type of control.

In the *BX-C* (3 – 58.8), homozygous mutations corresponding to lack of function cause segmental transformations to more anterior segments (cephalad transformations). Mutations which correspond to excess of function (derepression mutants, e.g., Contrabithorax, *Cbx*) cause transformations in the opposite direction (caudad transformations). Flies with extra doses of *BX-C* have no mutant phenotype. Lewis (1978) found that homozygous mutants in the Polycomb (*Pc*, 3 – 48) locus cause derepression phenotypes in all the segments under the control of the *BX-C*. In fact, heterozygous *Pc* flies occasionally exhibit a slight *Cbx* phenotype (a small patch of haltere tissue in the wing blade). This phenotype is more extreme and readily scored in flies with three doses of the *BX-C* (1*Pc*: 3*BX-C*). Mutants in the locus Regulator of bithorax (*Rg-bx*, 3 – 53.6) cause repression phenotypes (e.g., patches of wing tissue in the haltere) with a low penetrance. These are more extreme and show a higher penetrance in flies with one dose of the *BX-C* (1*Rg-bx*: 1*BX-C*).

The derepression phenotype of *Pc* disappears in homozygous *BX* mutants. This phenotype is enhanced by extra doses of the *Rg-bx* locus and disappears in flies hemizygous for the three loci (1*Pc*: 1*Rg-bx*: 1*BX-C*). Flies with three doses of the locus *Pc* or *Rg-bx* are phenotypically normal, even if they are heterozygous for *BX-C* deletions. The effect of *Pc* is strictly zygotic, whereas that of *Rg-bx* has a maternal component. Mutants of other loci tested differ in several ways from those two. These data are consistent with the

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model that *Pc* codes for a "repressor" whose effective concentration is modulated by the products of the *Rg-bx* (inducer) so that the unbound repressor determines the activity of the structural genes of the *BX-C*.

In the achaete-scute complex (*AS-C*, 1−0.0), mutations corresponding to lack of function cause removal of chaetae and sensillae all over the body. Achaete mutants preferentially remove microchaetae, while macrochaetae are removed by scute mutants. Derepression mutants in the complex (Hairy-wing, *Hw*) cause the appearance of extra-microchaetae in notum and wing. Extra doses of this complex cause no mutant phenotype. Mutants in the hairy locus (*h*: 3−26.5) in homozygous condition cause the appearance of extra-microchaetae, a derepression phenotype like that of *Hw*.

Deficiencies of the achaete region of the complex suppress the *h* phenotype of the homozygous *h* mutant. The *h* phenotype is enhanced when extra doses of *AS-C* are present. Moreover, flies heterozygous for *h*, a recessive mutant, have a dominant phenotype when extra doses of *AS-C* are present. Three doses of h^+ do not cause a mutant phenotype but suppress the derepression phenotype caused by *Hw* mutations in the *AS-C*. These genetic arguments support the idea that h^+ controls the activity of the *AS-C* by a negative type of control. In this model it is assumed that the modulation of the repressor concentration is caused by diffusible "inhibitors" between neighboring cells.

Results

Among 20 000 flies with three doses of the *BX-C* derived from ethyl methanesulfonate (EMS)-treated sperm, we found 15 flies with a derepression phenotype similar to that of *Cbx*. Of these, nine either failed to breed or did not transmit the phenotype to subsequent generations. The remaining six have a *Pc* phenotype, fail to complement with a deletion of *Pc* locus, and meiotically map to that locus, indicating that they are allelic to *Pc*. A cytological analysis failed to discover chromosome aberrations in any of them. After X-ray treatment we found, in 30 000 flies with three doses of *BX-C*, four flies with a *Cbx* phenotype; three of them failed to breed and one was shown to be a *Cbx* allele not associated with chromosome rearrangements.

In the *AS-C* experiment, half the flies carried one, and the other half two, extra doses of *AS-C*. The derepression phenotypes were found with equal frequencies in males (nine) and females (eight) with either one (nine) or two (eight) extra doses of the *AS-C*. In the EMS series, we scored 11 000 flies. We have found two yellow phenotypes (among the 5500 flies which carried one dose of y^+ in the *Dp(1:2)sc*[19]), and two flies with extra chaetae in a typical *h* pattern. Of the latter, one did not transmit the phenotype and the other was a *h* mutant, genetically proved to be associated with a T(1:3) translocation. Five flies (heterozygous for *TM2*) were found with a new pattern of chaetae: extra-macrochaetae appear in the notum as longitudinal rows in fixed positions, reminiscent of patterns in other Drosophilidae (García-Bellido, 1981a). However, this phenotype failed to appear in the offspring of these flies.

In 22 000 flies of the X-ray series we found nine yellow phenotypes (9:11 000) and six *h* phenotypes (of these three were isolated and proved to be allelic to *h*, none of them associated with chromosome rearrangements). In additon, 20 flies (19 heterozygous for *TM2* and one heterozygous for *TM1*) showed the extra-macrochaetae phenotype described above. Of these, 13 extra-macrochaetae mutants (including the one detected over *TM1*) were isolated. They all map to a locus in chromosome 3 (3−0) and fail to complement each other, but show a complex complementation pattern, ranging from lethality to almost wild-type phenotype. We have designated the locus extramacrochaetae *(emc)*. Out of nine analyzed cytologically, six are associated with chromosome rearrangements with breakpoints in 61C, one is a deletion from 61A to 61D, and two show no visible aberrations. All of them are fully recessive in male or female euploid flies. Since, even in the absence of extra doses of *AS-C*, *emc/TM2* flies have extra-macrochaetae, we conclude that the *TM2* balancer chromosome carries a weak allele of *emc*. However, of the 13 *emc* mutants found over *TM2*, two show a clear *emc* phenotype over a wild-type chromosome when extra doses of *AS-C* are present. These two alleles, a point mutant and the deficiency, probably corresponding to null alleles, show the most extreme phenotypes in combination with other *emc* mutants.

A similar genetic analysis to that described before with the *h* locus has been carried out in the case of *emc*. The derepression phenotype caused by *emc* is corrected with deficiencies of *AS-C*, in particular by those of the scute region of it, whereas it is enhanced with extra doses of *AS-C*. The phenotype of male and female mutant flies for *emc* have the same phenotypes for the same number of extra doses of the *AS-C*. These genetic arguments suggest that emc^+ codes for a "repressor" specific for the scute region of the *AS-C*, while h^+ is specific for the achaete region of it (García-Bellido, 1981a).

Repression phenotypes were only found and isolated in the *BX-C* experiment and in flies with one dose of the *BX-C:* 10 from the EMS and four from the X-ray series. These phenotypes comprise three mutants in the *BX-C* (one *bx*, and two *bxd* alleles), one allele of *Rg-bx*, and 10 which enhance the *Ubx* phenotype, two located in the X-chromosome, one located on the second, and seven on the third chromosome. None of these mutants have a phenotype in heterozygous flies with two doses of the *BX-C*.

Discussion

The efficiency of the screening method

The detection efficiency for all these phenotypes was similar within the X-ray and EMS series. Thus, *y* and *emc* (homozygous) were found at a frequency of 0.001 in the X-ray series and of ~0.0006 in the EMS series. The frequencies of *Pc*, *h*, and *emc* phenotypes detected in heterozygous condition are again similar and around 0.0005 in both the X-ray and EMS series. We were able to recover and verify the corresponding mutants in about half the phenotypically aberrant flies. As in any mutagenesis search, recovery is affected by several causes; among others: germ line mosaicism (especially in the EMS series), and rearrangements (especially in the X-ray series), leading to locus-dependent differential viability and fertility. Thus, it is possible that mutants in other loci with similar phenotypes may have failed to breed. Other types of mutants with strictly maternal effects, if they exist, will obviously have escaped our search.

Since the present protocol is designed to detect mutants in heterozygous condition, we may ask which are the limitations caused by the existence of loci that are lethal or sterile in one single dose (haplo-insufficient). It is known from the work of Lindsley *et al.* (1972) that the number of haplo-lethal loci in

the genome is very small. We do not have estimates of the haplo-sterile loci. However, we have analyzed the fertility of males and females with maximally derepressed *BX-C* and *AS-C*, using the most extreme *Pc*, *h*, and *emc* alleles in flies with two extra doses of either *BX-C* or *AS-C*. These flies are fertile. Thus, mutants in other genes causing the same derepression would have been recovered. If we assume similar mutability, scorability, and recoverability of mutants in other loci, the incidence of several alleles in *Pc*, *h*, and *emc*, suggest, on statistical grounds, that they are the only loci in the genome with such dose-dependent interactions. We have detected, in heterozygous condition, and isolated, six new *Pc* alleles, four new *h* alleles, and one *emc* allele (over *TM1*, half the analyzed flies). The probability of finding new loci with similar characteristics is low (for the *BX-C*, $P<0.002$ and for the *AS-C*, $P<0.05$).

We have tested dose-dependent relationships for known mutants which have derepression phenotypes similar or related to those of *Pc* and of *h* and *emc*. Of the former type, we have analyzed flies heterozygous for deficiencies for either the Antennapedia (Denell *et al.*, 1981) locus or the extrasex-comb (Struhl, 1981) locus and carrying three doses of the *BX-C*, and they do not show derepression phenotypes. We have similarly analyzed heterozygous flies with one and two extra doses of the *AS-C*, for, independently, Tuft, polychaetoyd, and polychaetus, with negative results. Also the combination of extra doses of the *AS-C* with several large deficiencies, adding up to 10% of the genome, have failed to show any derepression phenotype (unpublished results).

We have found several mutants that cause repression phenotypes in double heterozygous combination with a deletion of the *BX-C* (1:1 ratio). Some of them have been found to be allelic to other known loci (see Results). However, we have not attempted a systematic search for these mutants, and the ones found correspond to a small sample because we have only one allele in some of the loci. These mutants are interesting because they may be involved in the regulation of the bithorax complex, like *Rg-bx* (Capdevila and García-Bellido, 1981) or in the efficiency of its transcription (*Ubl*, a mutant of RNA polymerase II) (Mortin and Lefevre, 1981) or in the expression of the bithorax genes (see García-Bellido and Capdevila, 1978; Capdevila and García-Bellido, 1981; García-Bellido, 1981b).

Gene-dose titration analysis

Previous considerations suggest that the loci detected are the only loci of the genome that, in heterozygous condition, show interactions with extra doses of either the *BX-C* or the *AS-C*. Several questions derive from this conclusion. Firstly, what are the possible molecular bases of this gene-dose dependence? Several arguments strongly indicate that the expression of both the *BX-C* (Lewis, 1978; Capdevila and García-Bellido, 1978; García-Bellido, 1981b) and the *AS-C* (García-Bellido, 1981a) is under a negative type of control. The fact that derepression phenotypes caused by cis-dominant, reversible, mutations in the structural genes can be mimicked by lack of function mutations in other loci is more easily explained if the latter code for gene products which, directly or indirectly, lead to repression of the former. The fact that deletions of the structural genes are suppressors of the mutant regulatory genes suggests mutual interactions. Moreover, the fact that the phenotype of mutants in regulatory genes is exaggerated in individuals with extra doses of the structural genes, indicates very specific interactions.

In principle, these interactions could occur at the post-transcriptional level – the "regulatory" gene causing the specific decay of the products of the structural gene. However, the fact that extra doses of the regulatory genes do not have a phenotype on their own, not even in flies hemizygous for either the *BX-C* or *AS-C*, suggests rather that the interaction is not between the products of both genes. The study of flies doubly heterozygous for mutants in regulatory genes and for different types of mutants in the structural genes leads to a similar conclusion. For instance, the derepression phenotype of *Pc* is the same in wild-type flies and in flies heterozygous for null (amorphic) point mutant alleles of the *BX-C* and more extreme than in heterozygotes for deletions or rearrangements of this gene complex (Capdevila and García-Bellido, 1978, 1981; García-Bellido, 1981b). Due to "dose-compensation", genes in the X-chromosome have a differential transcriptional rate in euploid males and females, leading to equal amounts of gene products. These differential rates are maintained in duplications of these genes in autosomes, in such a way that, for the same number of duplications, males have more gene products than females. The fact that males and females heterozygous for either *h* or *emc* show the same derepression phenotype for the same number of extra doses of *AS-C*, which is dose-compensated, suggests that the genetic effect of the regulatory genes is on the structural genes rather than on their gene products. Thus, the available data are consistent with the interpretation that these regulatory genes act directly upon the structural genes, affecting their transcription. Obviously these considerations must remain speculative until molecular data can test them.

In this hypothesis, the amount of repressor-like products coded by these regulatory genes is limiting, both in absolute and in relative terms. As excess of products cannot cause more repression but its insufficiency leads to the derepression of the locus. It is reasonable to assume that regulatory genes acting in a negative type of control will have (effective) products in low concentration, otherwise the systems would not be capable of modulation, and repression would be constitutive. It follows that regulatory genes of the type found here should be sensitive to the number of copies of the structural gene they control. It is however questionable whether a 1:3 or 1:4 ratio is enough, as a rule, to detect, in the phenotype, its insufficiency. In fact, leaky mutants (hypomorphs) in these loci may have too great an amount of gene product to appear in our screen. Thus, there could be other genes in the genome with the same function which can only be detected with higher ratios or in homozygous condition. Those would not have been detected in the present experiments. However, the fact that we have found several alleles in such few loci, in fact one for each genetic system, bithorax, achaete and scute, is in accordance with the reasonable hypothesis that structural genes should not be regulated by many different regulatory genes at the transcriptional level; at least not in a negatively-controlled system.

It remains to be discussed how far dose-dependent interactions are indicative of regulatory relationships. Mutant phenotypes in double mutant combination (1:1 ratio) can be explained in several ways, as additive or synergistic gene insufficiency. Several loci are known and others have been reported here, that in heterozygous condition affect the expression of bithorax heterozygotes (Capdevila and García-Bellido, 1978, 1981; Mortin and Lefevre, 1981; Ingham and Whittle, 1980; Gans *et al.*, 1980), or achaete-scute mutants (Neel, 1941; Sturtevant, 1970). Other genetic tests are

necessary to classify their wild-type products as related to the activation of the structural gene, to its transcription, or to its expression in developmental terms (see García-Bellido and Capdevila, 1981; García-Bellido, 1981a, 1981b, for discussion). However, 1:3 gene dose interactions leading to derepression phenotypes of the structural gene are expected to be indicative of more specific relationships. These phenotypes cannot be easily explained by insufficient degradation of products of the structural gene, especially when the reciprocal combination, 3:1, does not lead to a repression phenotype. The wild-type allele of the interacting locus probably acts in a pathway leading to the repression of the structural gene. It must also act directly because, if separated by several steps, its insufficiency would have been corrected by the normal function of the intermediary genes in the pathway. Thus, mutants with similar derepression phenotypes, but not showing these gene-dose relationships, could correspond to genes that, although involved in the pathway, are removed some steps from the actual regulation. This test will, therefore, detect putative trans-regulatory genes, in a system under negative type control.

Mutants in regulatory genes of systems with a positive type of control would not be detected in 1:3 combinations because the activation of just one gene should give a normal phenotype. Perhaps they might fall in the class of 1:1 combinations giving repression phenotypes.

Searching for dose-dependent genes in this way helps to uncover functionally interrelated loci because of their haploinsufficient phenotype, even if they are homozygous lethal or linked to other recessive lethals. Obviously dose dependence in gene-dose titration experiments is not by itself a sufficient criterion for genes with regulator-structural relationship. Only subsequent analysis can permit the characterization of the individual loci and test the specificity of their action in the genetic system studied. However, mutual titration of wild-type genes and gene-dose dependence is more indicative of immediately related genes than the classical double mutant interactions. The search for dose-dependent genes, in this way, will allow us to progress from one locus to another in the genome to establish regulatory nets.

This type of genetic analysis hopefully will help to focus our attention on loci in the genome which are interesting candidates for a molecular analysis of their interactions.

Materials and methods

Mutagenesis

Mutations were induced in adult males by either X-rays (4000 R, Philips MG 151 Be, 100 kv, 15 mA 2 mm Al filter) or EMS (0.2 M; this dose induces ~ 1.2 lethal equivalents/major autosome). For description of mutants utilized in the screens see Lindsley and Grell (1968).

Screening protocol

Bithorax complex (BX-C). We searched for cephalad and caudad transformations in F_1 flies resulting from a cross between mutagen-treated wild-type males and females heterozygous for a *BX-C* deletion, carrying the dominant marker *Sb*, and a *BX-C* tandem duplication *(Df(3R)Ubx^{109}, Sb/Dp (3;3)P5)*. From this cross two kinds of flies emerge: *Sb* (one dose of the *BX-C*) and *Sb*$^+$ (three doses of the *BX-C*). The F_1 adults were scored under the dissecting microscope (x 30) for segmental transformations, and presumptive mutants were back-crossed to *Df(3)Ubx^{109}, Sb/Dp(3;3)P5* females in order to verify the mutation, amplify it, and confirm its dose-dependent phenotype. Subsequent crosses to autosomal balancers served to establish stocks for further analysis.

Achaete-scute complex (AS-C). We have searched for flies with more or fewer chaetae than wild-type among the offspring of a cross between mutagen-treated *y*; *Dp(1;2)sc^{19}/Dp(1;2)sc^{19}* males and *y/y*; *Dp(1;2)sc^{19}/SM5; TM1/TM2* females. The *Dp(1;2)sc^{19}* carries most wild-type alleles of the *AS-C* and of yellow, *y* (García-Bellido, 1979). *SM5, TM1,* and *TM2* are balancer chromosomes. In the F_1 flies, sons carry two or three, and daughters three or four, doses of *AS-C*. Any mutation induced, if located in the second or third chromosome is already balanced in the F_1. The subsequent steps to establish mutant stocks were as those described for the search in the bithorax system.

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